Original Article

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Development of a New HPLC Method for the Identification of Allicin and S-allyl Cysteine in Garlic (*Allium sativum L.*) Extracts

Sarımsak (*Allium sativum L.*) Ekstraktlarında Allisin ve S-allil Sistein Tayini için Yeni Bir HPLC Yönteminin Geliştirilmesi

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ABSTRACT

Introduction: In this study, a new high performance liquid chromatographic method was developed to determine the amount of allicin (AL) and s-allyl cysteine (SAC) in *Allium sativum L.* extracts.

Methods: In the method, C18 column (5 µm x4.6 mm x150 mm) was used as the stationary phase at 25 ºC and acetonitrile: water (70:30, v/v) mixture was used as mobile phase with 1 mL/min flow rate. Isocratic elution was applied. The injection volume was 20 µL. Measurements were carried out at 254 nm with ultraviolet detection. Retention times for AL and SAC were 1.1 and 2.4 min, respectively. The method was validated according to International Conference on Harmonization criteria.

Results: The limit of detection values for AL and SAC were 0.6 µg/mL and 1.5 µg/mL, respectively. The limit of quantitation values for AL and SAC were 2 µg/mL and 5 µg/mL, respectively. The linearity of the method was between 2-100 µg/mL and 5-30 µg/mL for AL and s-allyl cysteine, respectively. The developed method was also validated and applied to three different trade extracts.

Conclusion: This new method, which is quite fast, simple and economical, can be used in the analysis of *Allium sativum L.* extracts, which are named as black garlic in the contents of food supplements.

Keywords: Allicin, S-allyl sisteine, *Allium sativum L.,* HPLC-UV, validation

ÖZ

Amaç: Bu çalışmada, *Allium sativum L.* ekstraktlarındaki allisin (AL) ve s-allil sistein (SAS) miktarını belirlemek için yüksek performanslı yeni bir sıvı kromatografik yöntem geliştirilmiştir.

Yöntemler: Yöntemde C18 kolonu (5 µm x4,6 mm x150 mm) 25 ºC'de sabit faz olarak ve hareketli faz olarak 1 mL/dk akış hızında asetonitril: su (70:30, v/v) karışımı kullanıldı. İzokratik elüsyon uygulandı. Enjeksiyon hacmi 20 µL idi. Ölçümler 254 nm'de ultraviyole deteksiyon ile gerçekleştirildi. AL ve SAS için alıkonma süreleri sırasıyla 1,1 ve 2,4 dakika idi. Yöntem, Uluslararası Harmonizasyon Topluluğu kriterlerine göre valide edildi.

Bulgular: AL ve SAS için tespit değerlerinin limiti sırasıyla 0,6 µg/mL ve 1,5 µg/mL idi. AL ve SAS için miktar tayini değerlerinin sınırı sırasıyla 2 µg/mL ve 5 µg/mL idi. Yöntemin doğrusallığı AL ve SAS için sırasıyla 2-100 µg/mL ve 5-30 µg/mL arasındaydı. Geliştirilen yöntem ayrıca doğrulanmış ve üç farklı ticari ekstreye uygulandı.

Sonuç: Oldukça hızlı, basit ve ekonomik olan bu yeni yöntem, gıda takviyelerinin içeriklerinde siyah sarımsak olarak adlandırılan *Allium sativum L.* ekstraktlarının analizinde kullanılabilir.

Anahtar Sözcükler: Allisin, S-allil sistein, *Allium sativum L.,* HPLC-UV, doğrulama

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Introduction

Secondary metabolites, unlike primary metabolites, are not directly related to the essential vital activities of the plant. Adapting to the environment, pollination, competition, protection from pesticides and continuing its generations are the functions of secondary metabolites. Secondary metabolites are divided into three large classes: phenolic compounds, alkaloids and terpenes (1-2). Compounds containing sulphur, which have a similar effect to secondary metabolites, have been recently the subject of increased research (3).

Garlic (*Allium sativum L.*), belonging to the family *Liliaceae* (*Asphodelaceae*), is a bulbous flowering species of the genus Allium. Garlic, a spice preferred by people for many years, grows naturally in Central Asia and northeastern Iran and is widely used in the world. It is also utilized in Turkey as a food flavoring agent as well as a traditional medicine (4). The previous researches prove that garlic has anti-bacterial, anti-mycotic, anti-spasmodic, anti-diabetic, anti-oxidant, anti-cancer, anti-hyperlipidemic, hypotensive, vasodilator, anti-viral, fibrinolytic activity enhancing, thrombocyte aggregation slowing, anti-hepatoxic, and anti-atherosclerotic effects (5-10). It is used externally in wound healing and in the treatment of ear infections (11).

After the discovery of allicin (AL) (Figure 1) in 1994, many sulfur-containing compounds (allyin, s-allyl cysteine, diallylsulfide, allymercaptan) were identified in garlic. In recent studies, it has been reported that the amino acid s-allyl cysteine (SAC) (Figure 2), which contains a sulfur atom derived from garlic, has many biological activities. SAC is generally used as an alternative to AL in supplementary food preparations. The reason for this is that the AL has a pungent odor (12,13).

Up to date, to determine AL, SAC and bioactive sulfur compounds isolated from garlic (*Allium sativum* L.); high performance liquid chromatography-ultraviolet detector (HPLC-UV) (14,15), high performance liquid chromatography-electrochemical detector (16) and high performance liquid chromatography-mass spectrometry (17) methods have been used. However, there is no developed and validated method in the literature that enables the detection of AL and SAC in pharmaceutical preparations and nutraceuticals and dietary supplements. In addition, there is no method in the literature that quantitates AL and SAC simultaneously.

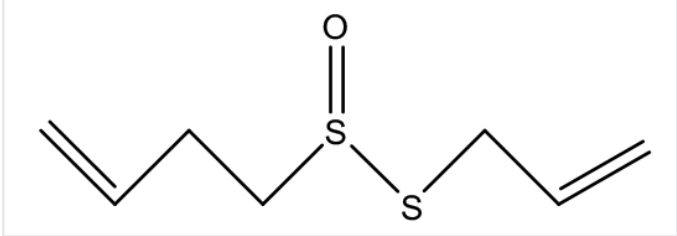

Figure 1. Chemical structure of AL
AL: Allicin

The aim of this study is to quantify the amount of AL and SAC in nutraceuticals and dietary supplements and extracts containing garlic; it is intended to validate an HPLC technique that will enable selective and sensitive analysis. The developed method does not require any derivatization and time consuming pretreatment procedure. Moreover, it is possible to carry out the separation process with a simply prepared mobile phase in isocratic elution profile rather than a complicated gradient procedure. The detection is also provided easily with UV detection that is used frequently in routine laboratories.

Method

Chemicals and Reagents

The AL and SAC were acquired from Sigma Aldrich, St. (Louis, Missouri, United States). Ethanol, methanol and acetonitrile of the HPLC category were obtained from Merck, Darmstadt, Germany. Water was treated through the Human Water systems made in Korea.

Solutions

The AL primary solution (10 µg/mL) concentration was prepared in ethanol: water mixture (7:3 v/v). SAC primary solution (100 µg/mL) concentration was prepared in ethanol: water mixture (10:10 v/v). These solutions were diluted with ethanol to give standard solutions of 2-100 µg/mL for both analytes.

Sample Preparation

In order to analyze AL and SAC in garlic (*Allium sativum L.*) extracts, various pretreatment procedures were carried out by dissolving the extracts in different solvents. Extracts were prepared by selecting the most suitable solvent where the dissolution was the best without interference from other components of the extract. Acetonitrile:water (7:3 v/v) was the most suitable solvent system to prepare the sample for chromatographic conditions. Because the sample was dissolved by this system as the best.

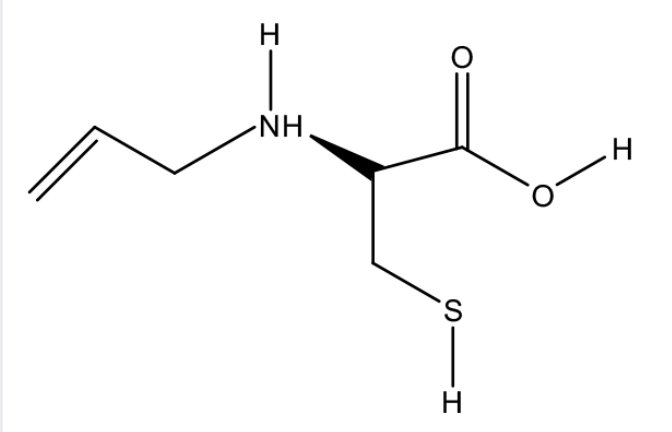

Figure 2. Chemical structure of SAC
SAC: S-allyl cysteine

Instrumentation

Spectrophotometric measurements of AL and SAC were made using the Shimadzu UV-160, a 1 cm glass cell spectrophotometer. HPLC tests were performed on a Shimadzu (Japan) LC 20 liquid chromatograph consisting of a LC-20AT pump, SIL AH-HT autosampler part, a SPD-20A HT UV spectrophotometric detector, which was set at 254 nm and CTO 10 AC column oven. The best separation was obtained as a result of experiments with various mobile phase and column types, different flow rates and different detector wavelengths.

Statistical Analysis

Power analysis was performed to determine the number of garlic extracts. The outcomes were presented as means ± standard deviation (n=3 per each test sample).

Results

Chromatographic process

Chromatographic conditions were performed at 25 ºC isocritically on a C18 (150 mm x4.6 mm x5 µm) (Shim-Pack, Shimadzu Corporations-Japan) column. The mobile phase consisted of a mixture acetonitrile and water (70:30, v/v). The experiment was done with a flow rate of 1 mL/min. 20 µL of the analytes was injected into the column.. The chromatograms of the *Allium sativum L.* extracts samples are given in Figure 3.

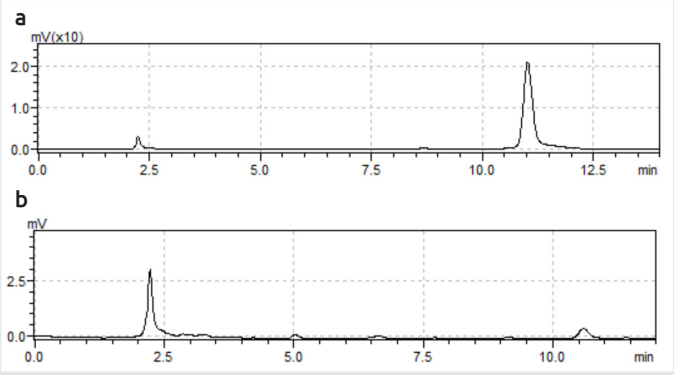

Figure 3. a) AL peak in garlic extracts, **b)** SAC peak in garlic extracts

AL: Allicin, SAC: S-allyl cysteine

The Calibration Graph

Calibration graph for AL was constructed by analysis of standard AL solutions at 8 different concentrations between 2-100 µg/mL. Calibration curve for SAC was prepared by analysis of standard SAC solutions at 5 different concentrations between 5-30 µg/mL. Regression equations of the AL and SAC were y=4762.2x-1367.6 (correlation coefficient =0.9959) and a y=874.61x-69.973 (correlation coefficient =0.9967) respectively.

Validation Parameters of the Method

The newly technique was validated according to the criteria presented by the International Conference on Harmonization (18).

Parameter of Sensitivity: The formula limit of detection (LOD) or limit of quantification (LOQ)=kSDa/b was used to compute the LOD and LOQ, where k=3 for LOD and 10 for LOQ, SDa was the standard deviation of the intercept, and b was the slope. As stated in Table 1; LOD and LOQ results for AL were 0.6 and 2 µg/mL, respectively.

Accuracy, Precision and Recovery: For the determination of AL in garlic extracts; quality control (QC) samples were prepared in several concentrations (2, 50 and 100 µg/mL) which could be categorized as low, medium and high concentration levels (n=3). For SAC determination; likewise, three different concentrations (5, 15 and 30 µg/mL) of QC samples were prepared (n=3). The accuracy was indicated by the recovery values and the accuracy of the recovery study was determined by the relative standard deviation (RSD) values of the recovery results in six repeated studies. The accuracy of the proposed method was quantified with standard addition technique by spiking QC specimens of standard AL and SAC solutions to garlic extracts including 15 µg/mL of AL and SAC. Absolute recovery of AL and SAC from garlic extracts, removal of AL and SAC from extracts, and comparison of peak areas got from the equal proportions of aqueous non-extracted AL and SAC solutions were examined and evaluated. The average absolute recoveries of AL and SAC were 87% and 90%, respectively. The calculated recovery was 101.55%. In order to determine the precision; three QC samples from each concentration were analyzed on the same day at different hours for intraday analysis and on 3 different days for interday analysis. In intraday tests, the RSD values for

Table 1. Results of analytical parameters for the proposed method

Parameter	Allicin	S-allyl cysteine
Linearity range* (µg/mL)	2-100	5-30
Regression equation	y=4762.2x-1367.6	y=874.61x-69.973
Slope ± SD	4762.2±1.57	874.61±1.23
Intercept ± SD	1367.6±5.66	69.973±0.38
Correlation coefficient, r^2	0.9959	0.9967
LOD (µg/mL)	0.6	1.5
LOQ (µg/mL)	2	5

*n=5 correspond to replicate analysis for each level.
SD: Standard deviation, LOD: Limit of detection, LOQ: Limit of quantification

AL and SAC were lower than 1.21 and 5.22, respectively. The RSD values of the inter-day results for AL and SAC were lower than 1.18 and 6.32, respectively. Table 2 and Table 3 indicate the recovery and RSD values of recovery.

Parameter of Robustness: Robustness studies were done by making minor changes to the method such as flow rate of the mobile phase and the column temperature. The mobile phase ratios were altered from (70:30 v/v) (acetonitrile-water) to 60:40 and 80:20; temperature was altered from 20 ºC to 30 ºC; and the flow rate was altered from 0.8 to 1.2 mL/min. These changes did not have a substantial effect on the system suitability parameters. RSD values were 4.73 and 3.76, respectively, as a result of the change of flow rate and mobile phase ratio. Table 4 illustrates the robustness finding.

Parameter of Stability: The working stability of AL and SAC substances was trialed in different storage conditions (at room temperature in the dark for 48 hours and under automatic sampling conditions for 4 ºC for 1 month) for long and short periods of time. In stability studies, it was found that the specimen were kept stable at room temperature for 48 hours and at 4 ºC for 1 month. For all of these trials, the highest RSD percent was 4.12 percent. AL and SAC were stable under all these conditions.

Application of the Method to the Determination of AL and SAC from Garlic *(Allium sativum* L.*)* Extracts

The solvent system that best dissolved AL and SAC from garlic *(Allium sativum* L.*)* extracts and was also the most suitable for chromatographic conditions was determined as ethanol:water (7:3). In order to analyze AL and SAC in *Allium sativum* L. extracts taken from 3 different commercial sources, it was developed after dissolving it in an ethanol:water (7:3) solvent system and filtering it through 0.45 µm membrane filters then studied under chromatographic conditions. The relative amounts of SAC contained in the extracts were determined as 68%, 60% and 58%, respectively. AL could not be detected in any of the analyzed extracts. This indicated that AL in these

Table 2. Results of recovery studies by standard addition method for S-allyl cysteine

	Amount present (µg/mL)[a]	Amount added (µg/mL)	Total amount found[b] (µg/mL) (mean ± SD)	Recovery (%)	RSD (%)	RSD of intraday variation (%)	RSD of interday variation (%)
		5	15.68±0.02	104.5	0.13	0.78	6.12
S-allyl cysteine	10	15	23.96±0.05	95.84	0.21	1.16	5.67
		30	41.5±0.11	103.75	0.27	1.18	6.32

Mean relative recovery=101.36
SD: Standard deviation, RSD: Relative standard deviation

Table 3. Results of recovery studies by standard addition method for allicin

	Amount present (µg/mL)[a]	Amount added (µg/mL)	Total amount found[b] (µg/mL) (mean ± SD)	Recovery (%)	RSD (%)	RSD of intraday variation (%)	RSD of interday variation (%)
		2	22.31±0.42	101.41	3.21	0.36	4.83
Allicin	20	50	68.9±0.35	98.43	3.56	1.45	4.78
		100	115.6±0.57	96.33	4.12	1.21	5.22

Mean relative recovery=98.72
SD: Standard deviation, RSD: Relative standard deviation

Table 4. Robustness of the method

Condition	Value	Recovery %	RSD %
Flow rate mL/min	0.8	94.26	4.73
	1.2	97.83	6.38
Mobile phase ratio (acetonitrile:aqueous phase)	60:40	103.76	3.76
	80:20	96.83	5.37
Column temperature	20	96.32	3.17
	30	103.24	5.28

n=3 for all quality control sample levels
RSD: Relative standard deviation

samples was completely fermented into SAC or that there was some unfermented AL below the LOD (0.6 μg/mL).

Conclusion

The medical effects of garlic (*Allium sativum L.*), especially antioxidant and antimicrobial activities, have been known for centuries. However, it is often not preferred due to the pungent smell of garlic. Therefore, consumption of fresh garlic by fermenting AL to SAC has become popular and fermented garlic preparations (extracts) have begun to appear in the market. In the literature, no method has been found that determines AL and SAC simultaneously. Existing methods for individual assays also include applications such as derivatization step and gradient elution mode. The method we have developed is quite simple, fast and low cost. The method does not require any derivatization reaction, it provides simple mobile phase with isocratic flow. A detection available in routine laboratories, such as UV detection, is used and has very sensitive and selective features.

Ethics

Ethics Committee Approval: This article does not contain any studies with human participants or animal performed by any of the authors.

Peer-review: Externally peer reviewed.

Authorship Contributions

Concept: G.T., B.C., Design: G.T., B.C., Data Collection or Processing: G.T., B.C., Analysis or Interpretation: G.T., B.C., Literature Search: G.T., B.C., Writing: G.T., B.C.

Conflict of Interest: No conflict of interest was declared by the authors.

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